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Mitochondrial DNA sequence patterns of Harbour porpoises (*Phocoena phocoena*) from the North and the Baltic Sea

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Abstract

To investigate genetic differentiation between harbour porpoises (*Phocoena phocoena*) of the North and the Baltic Sea, a total of 39 individuals were screened for sequence polymorphisms at a highly polymorphic part of the mitochondrial DNA control region. DNA was extracted from liver or skin samples of stranded animals. After PCR amplification and direct sequencing, 420 bp were scored. Nine haplotypes were found, differing from one another by one to four transitions. Haplotypes separated out into two clusters A and B by a specific nucleotide substitution. All Baltic harbour porpoises showed type A haplotypes, which was found only in 45% of the North Sea specimens. Genetic variation in terms of nucleotide and haplotype diversities was much lower in the Baltic Sea than in the North Sea population. Haplotype composition and nucleotide divergence suggest a colonization of the Baltic Sea several thousand years ago and a limited genetic exchange since then. The genetic differentiation between Baltic and North Sea populations of harbour porpoises is corroborated by published data both on skull character differences and enzyme polymorphisms.

Introduction

The harbour porpoise (*Phocoena phocoena*) is a small cetacean species inhabiting coastal waters in the Northern hemisphere (Nowak 1991; Kinze 1994). On a global scale, the populations of the North Atlantic, the North Pacific, and the Black Sea differ significantly in morphology, especially in skull characteristics, and have been described as three different subspecies *Phocoena phocoena phocoena*, *P. p. vomerina*, and *P. p. relicta* (Tomilin 1967; Yurick and Gaskin 1987; Amano and Miyazaki 1992; Kinze 1994). The subspecies status is corroborated by mitochondrial control region differentiation (Rosel et al. 1995). A further subdivision into local populations has been proposed due to limited migration and the incoherence of suitable habitat (Gaskin 1984; Kinze 1994). The population definition is controversial for the Eastern part of the North Atlantic: while some authors define three to four local populations (Gaskin 1984), others assume the entire area to be inhabited by a coherent population (Andersen 1972). However, there is some support for a separate Baltic population from skull character analyses (Kinze 1985) as well as from enzyme electrophoretic data (Andersen 1993).

From a population genetic point of view, the alternatives of population segregation vs. panmixia may be settled by a quantitative approach. Our comparison of the harbour porpoises from the North Sea and the Baltic Sea aims at estimating the amount and the direction of gene flow between those areas. In this context, mitochondrial DNA sequence patterns have been proven to be a suitable measure of genetic diversity within and among populations (cf. Avise 1994).

Material and methods

A total of 39 harbour porpoises stranded on the coast of the North Sea (n = 19) and the Baltic Sea (n = 20) were analysed (Fig. 1). Liver or skin samples were stored for 0–24 months at – 80 °C until analysis.

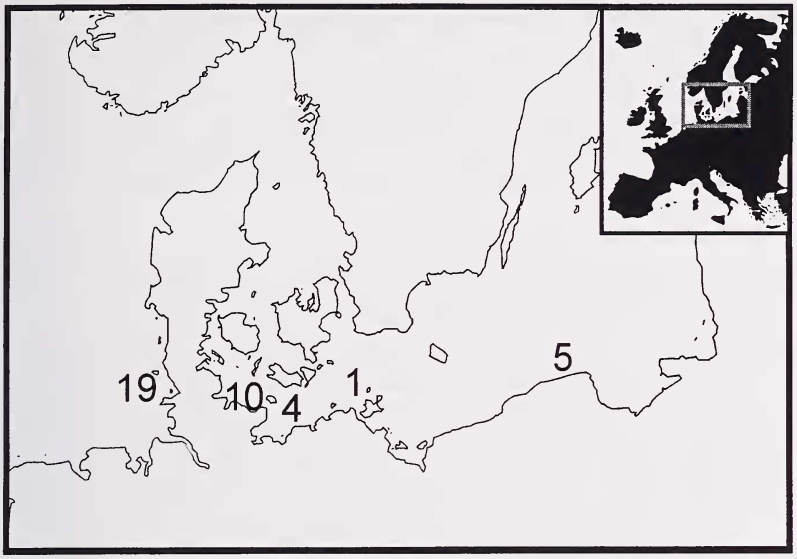

Fig. 1. Sampling localities (place of stranding) of the analysed harbour porpoise specimens. Numbers give sample size for each site.

Total DNA was exctracted from 100 mg of tissue using the SuperQuikGene DNA isolation kit (Analytical Genetic Testing Center, Denver, USA) according to manufacturer's instructions. The DNA was dissolved in a final volume of 100 µl Tris (pH 8.5). 5 µl DNA solution were used for an enzymatic amplification of a part of the mitochondrial control region via polymerase chain reaction (PCR), using the primers 5′-CACCACCAACACCCAAAGCT-3′ and 5′-CCTGAAGTAAGAACCAGATG-3′. These primers produce a product of 471 basepairs (bp), containing 45 bp of the Prolin-tRNA and 426 bp of the control region. The PCR-product contains the most variable part of the control region in Cetacea (Árnason et al. 1993). The following amplifcation profile was applied: After an initial denaturation step at 95 °C for 5 min, 40 cycles were carried out with a denaturation at 94 °C for 1 : 30 min, annealing at 51.9 °C for 1 : 15 min, and extension at 72 °C for 1 : 30 min, followed by a final extension at 72 °C for 2 : 30 min. The PCR-products were cycle-sequenced directly using the SequiTherm Cycle Sequencing Kit (Epicentre, Madison, USA) and the Digoxigenin-labelled oligonucleotide 5′-DIG-CACCAACACCCAAAGCT-3′ for 30 cycles, each with a denaturation at 95 °C for 30 s, an annealing at 53.2 °C for 30 s, and an extension at 70 °C for 1 min. Samples were run on a direct blot sequencing device (Richterich et al. 1989), and sequences were detected using an anti-Digoxigenin/alkaline phosphatase conjugate (Boehringer, Mannheim, Germany) and the chemiluminescent substrate CDP-Star (Tropix, Bedford, USA), following manufacturer's instructions.

Mitochondrial haplotypes were defined on the basis of 420 bp scored sequence of the control region and compared in terms of pairwise nucleotide divergence (Nei and Jin 1989), using the Neighbor-Joining-method (Saitou and Nei 1987) and the PHYLIP 3.5 c computer package (Felsenstein 1993). A published sequence of a Black Sea harbour porpoise was used as an outgroup (Rosel et al. 1995). A median graph of the relationships between mitochondrial haplotypes was determined according to Bandelt (1992). As measures of genetic variation within and among populations, mean nucleotide diversities within and between the two geographic areas were estimated (cf. Quinn and White 1987), and haplotype diversities within populations were calculated (Nei and Tajima 1981).

Results

The harbour porpoises analysed comprised nine different mitochondrial haplotypes as defined by sequence patterns of the Control Region. These were defined arbitrarily as Pho I to Pho IX, differing from one another by one to four transitions (Fig. 2, Tab. 1). An analysis of haplotype sequence divergence by the Neighbor-Joining procedure suggested two major clusters A and B, containing Pho I, Pho III, Pho VII, Pho VIII (cluster A), and Pho II, Pho IV, Pho V, Pho VI (cluster B) (Fig. 3). The haplotype Pho IX was sorted to none of these clusters and showed the highest similarity to a specimen from the Black Sea (BS), which served as an outgroup. As shown in a median graph, each haplotype could be derived from at least one other haplotype by a single point mutation, and the clusters A and B could be defined by a C and T, respectively, at position 198 (Fig. 4).

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          0000000000000000000000000011111111111111222222222333333333333333
          0011223334455566667888899901122223444469124567790011122555688 8
          6978371791302812474014568990201269018908971587074137890201535 67

NP        GTGTTGTATCTTAGTATTCACCTTCTGGCT-CCACACATCCACCCGCTCATAATCCCGGTCCT
BS        .C.A..C..TC..ACG.G.G......CAT.T.T--GTG...G..T..........T..CT.C
Pho I     .CAA.....TCC.ACGCG.G......C.T.T.T--GTG......T..........TA...TC
Pho II    .CAA.....TCC.ACGCG.G......C.T.T.T--GTG.T....T..........TA...TC
Pho III   .CAA.....TC..ACGCG.G......C.T.T.T--GTG......T..........TA...TC
Pho IV    .C.A.....TC..ACGCG.G......C.T.T.T--GTG.T....T..........TA...TC
Pho V     .CAA.....TC..ACGCG.G......C.T.T.T--GTG.T....T..........TA...TC
Pho VI    .C.A.....TC..ACGCG.G......C.T.T.T--GTG.T....T..........TAA..TC
Pho VII   .CAA.....TCC.ACGCG.G......C.T.T.T--GTG......T..........TAA..TC
Pho VIII  .CAA.....TC..ACGCG.G......C.T.T.T--GTG......T..........TAA..TC
Pho IX    .C.A.....TCC.ACGCG.G......C.T.T.T--GTG......T..........TA...TC
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Fig. 2. Sequence polymorphisms in the different haplotypes of harbour porpoise found in this study, as compared to specimens from the North Pacific (NP) and the Black Sea (BS) (np 1 and bs 1 from Rosel et al. 1995). Position numbers are indicated by three vertical digits.

Table 1. Number of different nucleotides (above diagonal) and pairwise nucleotide divergence in % (below diagonal) between mitochondrial haplotypes of harbour porpoise.

Pho	I	II	III	IV	V	VI	VII	VIII	IX
I	–	1	1	3	2	4	1	2	1
II	0.24	–	2	2	1	3	2	3	2
III	0.24	0.48	–	2	1	3	2	1	2
IV	0.71	0.48	0.48	–	1	1	4	3	2
V	0.48	0.24	0.24	0.24	–	2	3	2	3
VI	0.95	0.71	0.71	0.24	0.48	–	3	2	3
VII	0.24	0.48	0.48	0.95	0.71	0.71	–	1	2
VIII	0.48	0.71	0.24	0.71	0.48	0.48	0.24	–	3
IX	0.24	0.48	0.48	0.48	0.71	0.71	0.48	0.71	–

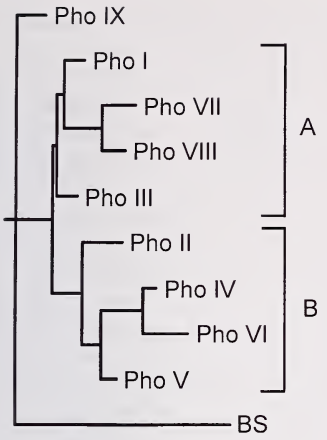

Fig. 3. Sequence divergence among haplotypes of harbour porpoises from North and Baltic Sea (Neighbor-Joining-tree, based on nucleotide divergence according to Nei and Jin 1989). A specimen from the Black Sea population (BS; cf. Fig. 2) served as an outgroup.

The geographic distribution of the haplotypes is given in table 2. The most common haplotypes Pho I and Pho VII were found in 62% of the animals. Overall mean nucleotide diversity was 0.37%, overall haplotype diversity was 0.80%. The genetic variation of the Baltic Sea population in terms of within population nucleotide diversity was less than 50% of the variation in the North Sea population, and also the haplotype diversity was considerably lower in the Baltic Sea (Tab. 2). The difference in the haplotype compositions of these two populations was highly significant ($p = 0.0001$; Fisher's exact test): all Baltic specimens showed cluster A haplotypes (95%-confidence limits: 83%–100%). These had only a frequency of 45% in the North Sea (95%-confidence limits: 26%–69%). The frequency of the most common haplotype Pho I had an increasing tendency from 26% in the North Sea through 40% in the Western Baltic Sea to 60% in the Eastern Baltic area (the last value was based on a very small sample size, however). As a measure of the divergence between the North Sea and the Baltic Sea population, net nucleotide diversity between them was estimated to be 0.13%. Net nucleotide diversity between the Western and Eastern subpopulation of the Baltic Sea was negligible (0.03%).

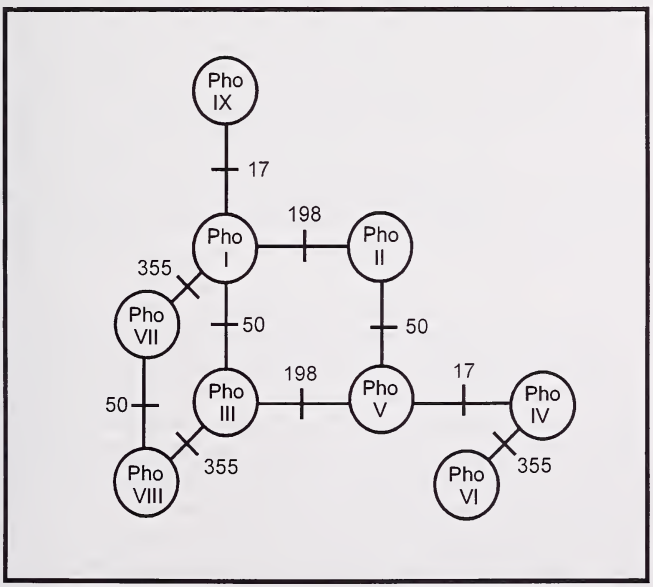

Fig. 4. Median graph of the relationships among haplotypes in terms of nucleotide substitutions. Note, that the transition at position 17 is included twice, i. e. as two different vectors, to allow a three-dimensial realisation of the graph.

Table 2. Geographic distribution of mitochondrial haplotypes, nucleotide diversity (π) and haplotype diversity (δ) within populations of harbour porpoise.

Haplotype	Total	North Sea	Baltic Sea	Western Baltic Sea	Eastern Baltic Sea
Pho I	14	5	9	6	3
Pho II	1	1			
Pho III	3	2	1	1	
Pho IV	4	4			
Pho V	2	2			
Pho VI	2	2			
Pho VII	10	2	8	8	
Pho VIII	2		2		2
Pho IX	1	1			
n	39	19	20	15	5
π(%)	0.36	0.42	0.18	0.15	0.23
δ	0.80	0.88	0.66	0.59	0.60

Discussion

For the total distribution range of the subspecies *P. p. phocoena*, i. e. the whole North Atlantic, mean nucleotide diversity in the control region was estimated to be 0.48%, and the West Atlantic population was found to be more diverse (0.58%) than the population of the East Atlantic (0.40%; all values recalculated from Rosel et al. 1995, using the formulae of Quinn and White 1987). Our value for North and Baltic Seas together (0.37%) was in good agreement with the value for the East Atlantic. Since all haplotypes except Pho I were only found either in the West or in the East Atlantic and net nucleotide diversity between these areas was high (0.28%; calculated for the combined data set of Rosel et al. 1995 and this study), gene flow between these areas appears very limited or even absent.

The predominance of the two ubiquitous haplotypes Pho I and Pho VII, which differed only by one nucleotide, suggests these types to be ancestral. This is corroborated by the results of a comparable study on eight harbour porpoises from Norway and the Danish Skagerrak, containing 4 times Pho I, once Pho VII, and three additional haplotypes, of which two could be derived from Pho I by a single transition (Rosel et al. 1995). Moreover, Pho I is the only haplotype that has been found both at the East and the West Atlantic coasts (Rosel et al. 1995). On the contrary, the characteristic transition at position 198, defining the cluster B haplotypes, was found neither on the West coast of the Atlantic nor in the Black Sea (Rosel et al. 1995) and may thus have arisen locally.

Considering the status of the Baltic harbour porpoise population, three alternative hypotheses concerning initial colonization and current extent of gene flow are to be discussed:

1. North Sea and Baltic Sea are completely panmictic (cf. Andersen 1972). We would expect the same haplotypes, occuring at similar frequencies, and similar nucleotide and

haplotype diversities within both populations. Net nucleotide diversity between the areas should be close to zero.

2. The Baltic Sea has been colonized once and then remained genetically isolated from the North Sea. Then, the Baltic Sea population could be expected to be genetically less diverse due to a persisting founder effect. The haplotype composition could be different as an effect of random genetic drift.

3. North Sea and Baltic Sea are inhabited by separated populations, but gene flow occurs occasionally. The Baltic Sea population might again be genetically less diverse, but its haplotype composition could represent a subset of the North Sea haplotypes.

The significant differences found in haplotype composition and nucleotide diversity prompt us to reject the first hypothesis of total panmixia: Cluster B haplotypes are absent in the Baltic Sea, and the nucleotide diversity of the Baltic Sea population is only half that of the North Sea population. When considering the second hypothesis of complete isolation between the two populations, we may invoke the concept of the molecular clock (cf. Avise 1994): Assuming that the part of the mitochondrial genome studied here may have diverged at a rate of about 15% per million years in an intraspecific comparison (Rosel 1992), the net nucleotide diversity suggests a divergence between Baltic and North Sea population about 8500 years ago. This coincides quite well with the age of the Baltic Sea as a brackish sea (*Litorina*-period; cf. Liedtke 1981). However, these values must be taken with caution, since both the divergence rates and the nucleotide diversities may contain stochastic errors. Nevertheless, these values provide some support for the second hypothesis. The haplotype composition of the two respective populations shows that all haplotypes found in the Baltic Sea were also present in the North Sea, except for Pho VIII, which was found only in the Eastern part of the Baltic Sea. This pattern could be explained by a colonization of the Baltic Sea by harbour porpoises with ubiquitous haplotypes (Pho I, Pho III, Pho VII). The low nucleotide and haplotype diversities in the Baltic Sea indicate a persisting founder effect. However, the Baltic population might be sufficiently large that it is not driven to haplotype uniformity by random genetic drift. Recent census data estimated a population size of about 50,000 specimens in the Baltic Sea, which is almost twice as high as in the Eastern part of the North Sea (Germany and Denmark combined; Kinze 1994; Hammond et al. 1995). It should be noted, however, that the majority of the Baltic Sea population is located in the Kattegat area (Hammond et al. 1995), which was not sampled in this study.

Considering the third hypothesis, the significant differences in haplotype composition between the two populations do on a first glimpse not support the interpretation of considerable gene flow between them. If we apply a model of the relation between the amount of gene flow and the frequency of exclusive genetic characters (i. e. exclusive haplotypes), we get a rough estimate of the number (Nm) of individuals migrating between separated populations per generation (Slatkin 1985). In this study, 55% of the North Sea and 10% of the Baltic Sea harbour porpoises have exclusive haplotypes, which gives an estimate for $Nm \approx 0.01$, i. e. one migrating specimen per 100 generations. Taking into account that Harbour porpoises are not fertile until the age of three to four years (Sørensen and Kinze 1993), this would correspond to only one migration event every several hundred years. This value apparently underestimates gene flow, since it would propose only about 10 to 20 migration events since the beginning of the *Litorina*-period of the Baltic Sea. The Nm value is possibly biased towards an underestimation for mainly two reasons: 1. There is some evidence that there is a seasonal pattern in the population structure within the North Sea (Andersen 1993; Kinze 1994). If there would be a seasonality both in the mitochondrial haplotype compositon in the North Sea and in the time of migration between North and Baltic Sea, the number of excluxive North Sea haplotypes may be overestimated, which consequently leads to an underestimate of Nm. 2. Mitochondrial DNA is maternally inherited. Thus, our Nm estimate does not include immigration

of males. Despite these cautions on the Nm estimate, it is an indication for only limited genetic exchange between the Baltic and the North Sea population of harbour porpoise.

The apparent genetic isolation of the Baltic Sea is surprising considering the known winter migration of Baltic harbour porpoises out of the Baltic (Dudok van Heel 1962), where they are likely to meet North Sea individuals (Kinze 1994). However, fertilization occurs in July and August (Møhl-Hansen 1954), when porpoises are in the Baltic Sea (Schulze 1987). Thus, the observed genetic segregation in the maternally inherited mitochondrial DNA between North and Baltic Sea could be explained by philopatry, assuming that at least females return to their area of birth for fertilization.

Differences between these areas are also suggested from investigations on isoenzymes, which are encoded in the nuclear genome and thus susceptible for gene flow in both sexes (Andersen 1993). Moreover, morphological characters indicate population differentiation (Kinze 1985). Thus, we conclude that the Baltic population of harbour porpoise has been genetically isolated after a postglacial colonization and gene flow into this population is a rare event.

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Zusammenfassung

Untersuchungen an der mitochondrialen DNA von Schweinswalen (Phocoena phocoena) aus Nord- und Ostsee

Um das Ausmaß genetischer Differenzierung zwischen der Nord- und der Ostsee-Population des Schweinswals (*Phocoena phocoena*) zu analysieren, wurden 39 Individuen auf DNA-Sequenzunterschiede in einem hochpolymorphen Abschnitt der mitochondrialen Kontrollregion untersucht. DNA wurde aus Leber- und Hautproben gestrandeter Tiere isoliert. Nach einer Amplifikation mit der Polymerase-Kettenreaktion (PCR) und direkter Sequenzierung der Amplifikate wurden 420 Basenpaare ausgewertet. 9 mitochondriale Haplotypen wurden gefunden, die sich jeweils an ein bis vier Nukleotidpositionen durch Transitionen unterschieden. Auf der Grundlage einer spezifischen Substitution wurden Haplotypen in zwei Gruppen (A und B) eingeteilt. Alle untersuchten Schweinswale aus der Ostsee zeigten Haplotypen der Gruppe A, die nur bei 45% der Nordseetiere gefunden wurden. Die genetische Variation, gemessen als Nukleotid- und Haplotypendiversität, war in der Ostseepopulation deutlich geringer als in der Nordseepopulation. Aufgrund der Frequenzen gefundener Haplotypen sowie der Nukleotiddiversität zwischen den Populationen ist davon auszugehen, daß die Ostsee vor einigen tausend Jahren durch Schweinswale besiedelt wurde. Nach dieser Besiedelung war der genetische Austausch mit der Nordseepopulation wahrscheinlich sehr gering. Die in dieser Untersuchung gefundene genetische Differenzierung zwischen Schweinswalen aus Nord- und Ostsee steht im Einklang mit Literaturangaben zu Unterschieden in Schädelmerkmalen und Enzympolymorphismen.

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